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RESEARCH ARTICLE

Cardiophysiological responses of the air-breathing Alaska blackfish to cold acclimation and chronic hypoxic submergence at 5°C

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ABSTRACT

The Alaska blackfish (*Dallia pectoralis*) remains active at cold temperatures when experiencing aquatic hypoxia without air access. To discern the cardiophysiological adjustments that permit this behaviour, we quantified the effect of acclimation from 15°C to 5°C in normoxia (15N and 5N fish), as well as chronic hypoxic submergence (6–8 weeks; ∼6.3–8.4 kPa; no air access) at 5°C (5H fish), on *in vivo* and spontaneous heart rate (f_H), electrocardiogram, ventricular action potential (AP) shape and duration (APD), the background inward rectifier (I_{K1}) and rapid delayed rectifier (I_{Kr}) K^+ currents and ventricular gene expression of proteins involved in excitation–contraction coupling. *In vivo* f_H was ∼50% slower in 5N than in 15N fish, but 5H fish did not display hypoxic bradycardia. Atypically, cold acclimation in normoxia did not induce shortening of APD or alter resting membrane potential. Rather, QT interval and APD were ∼2.6-fold longer in 5N than in 15N fish because outward I_{K1} and I_{Kr} were not upregulated in 5N fish. By contrast, chronic hypoxic submergence elicited a shortening of QT interval and APD, driven by an upregulation of I_{Kr}. The altered electrophysiology of 5H fish was accompanied by increased gene expression of *kcnh6* (3.5-fold; K_v11.2 of I_{Kr}), *kcnj12* (7.4-fold; K_{ir}2.2 of I_{K1}) and *kcnj14* (2.9-fold; K_{ir}2.4 of I_{K1}). 5H fish also exhibited a unique gene expression pattern that suggests modification of ventricular Ca^{2+} cycling. Overall, the findings reveal that Alaska blackfish exposed to chronic hypoxic submergence prioritize the continuation of cardiac performance to support an active lifestyle over reducing cardiac ATP demand.

KEY WORDS: Action potential, Electrocardiogram, Excitation–contraction coupling, Heart, K^+ channels, Temperature

INTRODUCTION

Freshwater fish that reside in the north-temperate zone experience pronounced seasonal variation of ambient temperature and oxygen availability (Eliason and Anttila, 2017; Stecyk, 2017; Vornanen, 2016). Consequently, in winter, cardiac physiology must be adjusted to accommodate the cold temperature-driven effects on contractility, blood viscosity and associated vascular resistance to ensure the efficient transport of respiratory gases, nutrients, waste products and signalling molecules of the endocrine system among tissues via the circulatory system (Young and Egginton, 2011). In addition, in winter, cardiac electrical excitability must be adjusted to coincide with temperature-dependent reductions in heart rate (f_H), while maintaining cardiac excitability and protecting against cardiac arrhythmias that can be induced by cold temperature and oxygen deprivation (Vornanen, 2017).

The physiological strategy employed by fishes in response to cold temperature acclimation (or acclimatization) varies among species and is dependent on thermal preference, thermal tolerance and the ability to withstand oxygen deprivation. Some species exhibit compensatory phenotypic changes against the depressive effects of cold temperature that allow for the continuation of an active lifestyle in winter (reviewed by Vornanen, 2011b, 2016, 2017). By comparison, species that experience prolonged periods of anoxia during the winter months, such as the anoxia-tolerant crucian carp (*Carassius carassius*), must prime physiological processes to conserve ATP, making positive compensatory changes detrimental (Hochachka, 1986). Accordingly, for the crucian carp, as well as for other anoxia-tolerant ectothermic vertebrates that are deprived of oxygen in winter, namely freshwater turtles of the genus *Chrysemys* and *Trachemys*, cold exposure serves as an important cue to reduce activity, metabolic rate and subsequently cardiac activity in anticipation of winter hypoxic and/or anoxic conditions (Couturier et al., 2019; Farrell and Stecyk, 2007; Herbert and Jackson, 1985; Jackson, 2000; Stecyk et al., 2008, 2017; Stecyk, 2017; Vornanen et al., 2009). The phenomenon is termed inverse thermal acclimation.

Nevertheless, some cardiophysiological responses to cold are not mutually exclusive to an overwintering strategy. For instance, a pronounced and shared response between cold-active and cold-dormant species with cold acclimation and acclimatization is a prominent shortening of action potential duration (APD) (Abramochkin and Vornanen, 2015, 2017; Filatova et al., 2019; Galli et al., 2009; Hassinen et al., 2014, 2008b; Haverinen and Vornanen, 2009; Shiels et al., 2006; Stecyk et al., 2007; Vornanen, 2016, 2017; Vornanen et al., 2002). The mechanism underlying the shortening of APD is an upregulation of the main cardiac K^+ currents I_{K1} and I_{Kr}, especially I_{Kr}, which is considered to play the most important role in the control of APD in fish myocardium (Hassinen et al., 2008a). I_{K1}, an outward K^+ current via background inward rectifier K^+ channels, is primarily responsible for maintaining a stable resting membrane potential (V_{rest}) and terminal repolarization of the action potential (AP), but is not thought to be present during the AP

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plateau. I_{Kr}, a repolarizing K^+ current via rapid delayed rectifier K^+ channels, gradually develops during the plateau phase of the AP, conducts outward current at more positive voltages than I_{K1}, and is thus important in balancing Ca^{2+} influx and contributing to the plateau of the cardiac AP. The augmented K^+ conductances serve to partially (in cold-dormant species) or fully (in cold-active species) compensate for the depressive effect of cold temperature on APD.

The Alaska blackfish is a facultative air breather that relies on air breathing in summer to support the energetic demands of foraging and reproduction in waters that are dense in vegetation, stagnant and hypoxic (Lefevre et al., 2014; Ostdiek and Roland, 1959; Scholander et al., 1953). However, unlike the majority of air-breathing fish, which reside in the tropics and have year-round access to atmospheric oxygen (Graham and Wegner, 2010), the Alaska blackfish is endemic to some of the coldest places on Earth: the tundra wetlands of western and northern mainland Alaska, the Bering Sea islands and Eastern Siberia (Campbell and Lopéz, 2014; Campbell et al., 2015). Consequently, in winter, the fish are forcibly submerged beneath thick layers of ice and snow, which not only precludes air breathing but also results in aquatic hypoxia (Haynes et al., 2014; Lefevre et al., 2014; Leppi et al., 2016). Indeed, the fish inhabit year-round shallow (<4 m depth) lakes that have oxygen partial pressures (P_{O2}) ranging from 9% to 75% air saturation (~1.9 to 15.8 kPa) in winter (Leppi et al., 2016). Yet, intriguingly, overwintering Alaska blackfish remain active. If local conditions become untenable, the fish must migrate through the shallow drainage ditches that interconnect the numerous lakes and ponds found on the Arctic tundra to seek out less hypoxic and deeper unfrozen refugia to ensure survival (Haynes et al., 2014; Leppi et al., 2016).

In concordance with the idiosyncratic overwintering ecology of the Alaska blackfish, previous studies have revealed that the fish displays a combination of down-regulatory, cold-compensatory and acute and perhaps direct metabolic and cardiac modifications with acclimation to 5°C from 15°C in normoxia (Kubly and Stecyk, 2015, 2019; Lefevre et al., 2014). Down-regulation was evidenced by the absence of cold-induced ventricular hypertrophy, an 8-fold reduction of peak ventricular L-type Ca^{2+} current (I_{Ca}) density and a prolongation of the relaxation phase of ventricular contraction that was not reversed with acute warming. Cold compensation was evidenced by alterations of the I_{Ca} Ca^{2+}-dependent and voltage-dependent inactivation properties that serve to limit the reduction of total Ca^{2+} transferred through the channel, an enhanced inotropic responsiveness to adrenergic stimulation and the persistence of an impressive capability to increase metabolic rate 5- to 8-fold at cold temperature. Acute and perhaps direct responses to lowered temperature were evidenced by decreases in ventricular contraction kinetics and whole-animal standard total oxygen consumption with Q_{10} values near 2. Nevertheless, the cardiophysiological adjustments that allow the Alaska blackfish to remain active at low temperature when experiencing aquatic hypoxia without air access remain enigmatic.

To fill this information gap, we comprehensively examined how the heart of the Alaska blackfish responds to the ambient temperature and oxygen conditions the fish experiences in winter. Our integrative approach measured several cardiac parameters spanning multiple levels of biological organization of 15°C-acclimated normoxic (15N) fish, 5°C-acclimated normoxic (5N) fish and 5N fish that were subsequently exposed to chronic aquatic hypoxia (P_{O2} of ~6.3–8.4 kPa) for 6–8 weeks without access to atmospheric oxygen (5H fish). Specifically, we measured *in vivo* resting f_H and cardiac electrical activity by electrocardiogram (ECG), *in vitro* spontaneous f_H, ventricular AP shape and APD from spontaneously beating cardiac tissue preparations by sharp electrode, I_{K1} and I_{Kr} of isolated ventricular myocytes by patch clamping and the ventricular gene expression of 15 cellular components of excitation–contraction coupling, including the pore-forming subunits of I_{K1} and I_{Kr} channels, by quantitative PCR (qPCR) (Table 1). *In vitro* spontaneous f_H, ventricular APs and K^+ currents were measured at the acclimation temperature of the fish, as well as at the common test temperature of 10°C to distinguish effects of cold acclimation from acute and perhaps direct effects of ambient temperature on the rate of these physiological processes. We hypothesized that, consistent with past studies, 5N fish would exhibit a mix of cold-compensatory, down-regulatory and acute and perhaps direct cardiophysiological responses, whereas 5H fish would exhibit cardiophysiological changes reflective of reduced cardiac ATP demand.

MATERIALS AND METHODS

Experimental animals

Animals were collected under appropriate Alaska Department of Fish and Game permits (SF-2014-062, SF-2015-025 and SF-2016-30d) and the University of Alaska Anchorage (UAA) Institutional Animal Care and Use Committee approved all procedures (406888, 421896, 421899, 852440, 852441 and 852442). Eighty-one Alaska blackfish (*Dallia pectoralis* T. H. Bean 1880) with a mean (±s.d.) body mass of 36.5±12.3 g were utilized. Fish of both sexes were collected in spring and summer using minnow traps from local waters (Little Campbell Lake, Anchorage, AK, USA; Duck Hunter's Training Pond, Palmer, AK, USA) and were maintained indoors under a 12 h:12 h light:dark photoperiod in 300 l fibreglass aquaria (190×40×40 cm W×H×D, 300 l) that contained recirculating, dechlorinated and fully aerated water. Fish were initially held at their natural habitat temperature, which ranged between 10 and 16°C.

Temperature acclimation

Fish were randomly divided into aquaria (two aquaria per acclimation temperature) and exposed to 15 or 5°C by incrementally decreasing (1°C per day) or increasing (0.5°C per day) water temperature. Fish were then held at 5 or 15°C for ~6 months (from October until March) prior to experimental measurements or chronic exposure to aquatic hypoxia without air access. The temperature of each aquarium was regulated with a cooler/heater system (Teco-TR20, Senkor Group, Inc., Terrell, TX, USA).

Chronic exposure to aquatic hypoxia without air access

At the conclusion of the temperature acclimation period, a subset of the 5N fish were exposed (split across two aquaria) to hypoxia and prevented from breathing air at 5°C (5H fish). For these fish, access to the atmosphere was first restricted by suspending plastic grating (1 cm^2 holes) 5 cm below the water surface. After 1 week, P_{O2} of the water was progressively lowered (4 days at ~14.7 kPa, 4 days at ~12.6 kPa, 6 days at ~10.5 kPa) and then maintained between ~6.3 and 8.4 kPa for 6–8 weeks. The final level of hypoxia was selected to represent an approximate average of the range of dissolved oxygen levels Alaska blackfish experience in winter (~1.9–15.8 kPa) (Haynes et al., 2014; Leppi et al., 2016). The level of hypoxia was also selected so as to not endanger the life of the fish. The critical oxygen partial pressure (P_{crit}) of 5N Alaska blackfish ranges from 4.3 to 7.5 kPa, and 5N fish that are exposed to a lower level of aquatic hypoxia (P_{O2} of 3.3 kPa) and deprived of air access rapidly (within 13 h of exposure) lose equilibrium (Lefevre et al., 2014).

Water P_{O2} was measured and maintained at appropriate levels using an OXYREG control unit, galvanic oxygen probe and solenoid valve gas controller that regulated the bubbling of the water with 100% N_2 (Loligo Systems, Tjele, Denmark). The oxygen

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Table 1. Gene targets, primers utilized for partial cloning, and the sequences, priming efficiencies and Cq values of the qPCR primers averaged from all qPCR reactions

Gene	Encoded protein	Accession no.	Primers for partial cloning	Primers for qPCR	Eff	Cq
atp1a1	ATPase, Na^+/K^+ transporting, alpha 1 polypeptide (Na^+/K^+-ATPase)	MT211888	F CTCTGAGGGCAACGAGACTG R ATACTGGCGAAGAACGCTGT	CCCTTCTTATTCTTCATCATTGCGA GAGTTTCTGGGCTGTCTCTTCA	2.02±0.11	23.4±1.2
atp2a2	ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2 (SERCA2)*	MT211889 MT211890	F CGATGAGATCACCGCCATGA R AGGGACATGGAGAGGCAGAT	AACAACATGAAGCAGTTCATCCG GCTTGTTCATGATGTCCAGGTC	2.04±0.12	22.1±1.3
cacna1c	Ca^{2+} channel, voltage-dependent, L-type, alpha 1C subunit ($Ca_v1.2$)	MT211891	F CGTGGTCGTAGGGTTGTTCA R GTCCAGCCCTCCATGGTGATA	CTCAGATTGGTGTCTGGAGTACC CGATGCATCTTCCCCATAAACAG	1.96±0.08	24.4±1.2
kcnh6	I_{Kr}-producing rapid voltage-gated (delayed rectifier) K^+ channel alpha subunit (ERG-2; $K_v11.2$)	MT211892	F AGACCGCTCTGACACAGTTG R GAGCACAGTGTAGTGGCCTT	TGGTCCCTGTGAAGAACGAG TCAACTTCAACCTGCGACCC	1.94±0.08	24.5±1.4
kcnh7	I_{Kr}-producing rapid voltage-gated (delayed rectifier) K^+ channel alpha subunit (ERG-3; $K_v11.3$)	MT211893	F CTCAGATTGACGAGGGGTCG R TTTCCGTAACCGGAGAAACAG	CAAAATCGACTCTCCAGGGGAA GAATGCAGATGTTAGGTGACGC	1.92±0.02	32.0±1.8
kcnj2	I_{K1}-producing cardiac inward rectifier K^+ channel subfamily J, member 2 ($K_{ir}2.1$)	MT211894	F GTCTTCCAGTCCATCGTGGG R CATGCAGGATGGTGATGGGT	GCCAAGCCGAAGAAGAGGAAC ACTGTCAAAGCCCACGTTGATG	1.91±0.02	28.4±1.7
kcnj12	I_{K1}-producing cardiac inward rectifier K^+ channel subfamily J, member 12 ($K_{ir}2.2$)	MT211895	F CGCAACCGCTTTGTCAAGAA R CATGCAGGATGGTGATGGGT	GAAGAATGGCCAGTGCAACATT ACCCAGAAGATGACACCAAACA	1.92±0.02	33.5±1.0
kcnj14	I_{K1}-producing cardiac inward rectifier K^+ channel subfamily J, member 14 ($K_{ir}2.4$)	MT211896	F TAGCTTCACTGCTGCCTTCC R GGGCCACCTCGTTCTCATA	TCCATTGGTTACGGCTTTCGGA TCATGCAGAGTTTCCCGTCTCT	1.93±0.03	27.9±1.1
kcnj3	G protein-activated inward rectifier K^+ channel 1 (GIRK1; $K_{ir}3.1$)	MT211897	F GTCCCAGCGTTCTCTGTTGA R GCAGTTTGGCAGGTAGGTTG	ACTTGACTCCATCACTGTTCCC TAGAGGGATCCTGACTGTGGTT	1.92±0.02	31.2±1.4
kcnj5	G protein-activated inward rectifier K^+ channel 4 (GIRK4; $K_{ir}3.4$)	MT211898	F CTACGTGGTCAACTGGCTGT R GGGCGTGTTGGTCTTGTAGA	AAGCTGATCCGCTCACAACA GGGGAAACCAGGAAGAGTCG	1.92±0.02	30.5±2.1
kcnq1	I_{Ks}-producing slow voltage-gated (delayed rectifier) K^+ channel alpha subunit ($K_v7.1$)	MT211899	F AGGAAACCTGTTCTGCCTCG R ATTCGTGCCAGGGTTAAGGG	TTGTCTCCAAGAAGAGGTTCCAG TTCAGGTGTCCTTGGGAATACTG	1.94±0.03	27.1±1.5
pln	Phospholamban	MT211900	F CGTCCCAGATCGAGGTGAAC R CGATGATGTAGATGAGCAGCA	CCACAGACCAAACGTAACCTAC GCAGATGAGGATGAGGGAGA	1.94±0.03	34.1±0.8
ryr2	Cardiac muscle ryanodine receptor-Ca^{2+} release channel*	MT211901 MT211902	F CCGAAGGCTTTGGGAACAGA R CTCGTCCATGTGACCGTGAA	TTTGTCCTGGAACAGTCTCTCTC CTTCACCTGTTGTGTCTTCTTGC	1.90±0.03	27.7±1.4
scn4a	Na^+ channel, voltage gated, type IV alpha subunit ($Na_v1.4$)	MT211903	F CACCGTCAGCTGGAACATCT R TCGCCACACTGAAGTTCTCC	AGCTGGAACATCTTCGACCTGG GGGTCCTTATTCCTTTGGCTCC	1.94±0.02	30.8±1.0
slc8a1	Solute carrier family 8, member 1 (Na^+/Ca^{2+} exchanger; NCX)	MT211904	F CTACATGTTCCTGGGCGTGT R TCCCTCGGTGAACTCGTAGT	GATCACCATCAAGAAACCCAACG GCGATGATGACGAACATGTTGAA	1.92±0.03	26.6±1.5
mw2060	n/a; external RNA control from the cyanobacterium *Microcystis* cf. *wesenbergi*	DQ075244	F n/a R n/a	GTGCTGACCATCCGAG GCTTGTCCGGTATAACT	1.89±0.02	27.8±1.9

Eff, priming efficiency; Cq, quantification cycle; F, forward primer; R, reverse primer; n/a: not applicable. Values are means±s.d. qPCR amplicons ranged from 60 to 250 bp.

*Two isoforms of *atp2a2* and *ryr2* were found. The qPCR primers were designed to conserved regions to capture both isoforms.

probes measured dissolved oxygen as percentage of air saturation and were calibrated daily. Calibration to 100% was achieved by bubbling a small volume of 5°C water with atmospheric air, whereas calibration to 0% was attained according to the manufacturer's protocol, by short-circuiting the system and setting the resulting signal output as the 0% value. Oxygen levels in the hypoxic tanks were also confirmed once or twice daily using a fibre optic FDO 925 oxygen probe and Multi 3410 meter (WTW, Weilheim, Germany).

Animal husbandry

15N, 5N and 5H fish were fed *ad libitum* every 2–3 days with bloodworms (Brine Shrimp Direct, Ogden, UT, USA), but food was withheld 24 h prior to experimental measurements. To maintain levels of nitrite, nitrate and ammonium below recommended levels (Tetra EasyStrips, Tetra, Blacksburg, VA, USA), two-thirds of the water in each aquarium was changed once a week. To prevent 5H fish from gaining air access and/or experiencing increased water

oxygen levels during water changes, water changes were accomplished with as little disturbance to the water column as possible and without lowering the water level below the level of the submerged grating. Additionally, the fresh water that was added to the 5H fish was pre-bubbled with 100% N_2 to have a P_{O2} between 6.3 and 8.4 kPa. Moreover, the behavioural response of the fish to seek the bottom and hide in the moss present in each aquarium when disturbed precluded the fish from nearing the water surface during water changes.

Recording and analysis of ECG from live fish

ECG recordings were obtained from unrestrained and un-anaesthetized fish using established methods (Campbell et al., 2004; Tikkanen et al., 2017). Fish were anaesthetized with buffered tricaine methanesulfonate (MS-222; 0.2 g l^{-1}+0.2 g l^{-1} $NaHCO_3$; Sigma Aldrich, St Louis, MO, USA) until opercular movements ceased. Fish were then weighed, placed ventral-side up on an operating table and the gills continuously irrigated with an ice-cold oxygenated, lower dose of buffered MS-222 solution (0.1 g l^{-1}+0.1 g l^{-1} $NaHCO_3$). For 5H fish, the MS-222 solution was bubbled with 100% N_2 to achieve a P_{O2} between 6.3 and 8.4 kPa. Two recording electrodes made of 40 cm long, 0.23 mm diameter, 7-stranded Teflon-coated wire (A-M Systems, Sequim, WA, USA) were inserted obliquely from the ventral side of the fish laterally close to the pericardium. The electrodes were secured to the animal with 3-0 gauge silk thread sutures at the site of insertion and near the dorsal fin. Following the surgical procedure, which lasted an average of 16 min, individual fish were placed into horizontal Plexiglas cylindrical chambers that were immersed within a larger tank that contained water at a temperature and oxygenation level that matched the acclimation condition of the animal. The temperature and oxygen level of the larger tank were regulated with a Teco-TR20 cooler/heater system (Senkor Group, Inc.) and OXYREG system (Loligo Systems), respectively. 15N and 5N animals were permitted air access within the Plexiglas chambers, whereas the chambers were completely submerged for the 5H fish, precluding air breathing. In all instances, the chambers were enclosed with mesh at both ends to allow circulation of oxygenated (for 15N and 5N fish) or hypoxic (for 5H fish) water and covered with dark plastic to minimize stress to the animals. ECG signals were recorded continuously for 1 h immediately following instrumentation, and thereafter for 1 h every 24 h for 5 consecutive days commencing at 14:00 h each day. ECG leads were connected to FE136 Animal Bio Amps (ADInstruments, Colorado Springs, CO, USA) and the signals were digitized at 1000 Hz and recorded to a computer with a PowerLab 8/35 data acquisition system (ADInstruments).

f_H was calculated off-line from the RR intervals of the ECG recordings using Labchart 8 software (ADInstruments). A minimum of 10 min from each recording period that had clean ECG traces and coincided with when fish were calm and not air breathing (for 15N and 5N fish) was selected for analysis. For recordings obtained at 120 h, at least 5 electrical impulses per fish displaying good quality P, QRS and T waves were quantified using the ECG Analysis module of Labchart 8 software (ADInstruments) and averaged per fish. Manual confirmation of automatically calculated values and quantification of ECGs was also conducted as necessary. The following parameters were quantified: the P-wave duration (start of P to end of P), which represents depolarization of the atrium; the PR interval (start of P to start of QRS), which represents the delay between atrial and ventricular depolarization; the QRS complex duration (start of Q to end of S), which indicates the time required for AP depolarization to propagate through the ventricle; and the QT interval (start of Q to end of T), which represents the average duration of the ventricular AP.

Two-way repeated measures (RM) analysis of variance (ANOVA) were used to determine statistically significant differences in *in vivo* f_H within and between acclimation groups over time. One-way ANOVA were employed to determine statistically significant differences in ECG parameters among acclimation groups. When appropriate, *post hoc* comparisons were determined using Student–Newman–Keuls analysis. Differences were considered statistically significant when $P<0.05$.

Recording and analysis of spontaneous f_H and ventricular APs from cardiac tissue preparations

Intracellular APs were measured from the ventricle of spontaneously beating cardiac tissue preparations. The entire heart was excised, including the sinus venosus, atrium and ventricle, taking care not to damage the pacemaker region (Haverinen and Vornanen, 2007). The tissue was rinsed in ice-cold physiological saline and the ventricle medially opened, spread and gently fixed with insect pins to the Sylgard-coated bottom of a 20 ml, water-jacketed tissue chamber filled with physiological saline (see below) and regulated at the acclimation temperature of the animal (i.e. 15°C for 15N fish; 5°C for 5N and 5H fish) with a refrigerated circulating water bath (VWR, Radnor, PA, USA). A hooked insect pin connected with 3-0 surgical silk to a FT03C force displacement transducer (Grass Instruments, West Warwick, RI, USA) was attached to the edge of the ventricle to monitor myocardial contractions. The composition of the physiological saline was (in mmol l^{-1}): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 Hepes and 10 glucose. Saline pH was titrated to 7.6 with NaOH at 21°C using an Orion Star A211 pH meter with an Orion ROSS Ultra Glass Triode pH/ATC combination electrode (Thermo Fisher Scientific, Waltham, MA, USA) and was allowed to change with temperature (temperature coefficient of Hepes: −0.014 $°C^{-1}$); pH was 7.68 at 15°C, 7.75 at 10°C and 7.82 at 5°C. The saline was bubbled continuously with 100% O_2 for all acclimation groups. This was done to ensure comparison among the three exposure groups under identical experimental conditions and because the level of hypoxaemia in 15H fish was unknown.

Hearts were allowed a 30–45 min stabilization period before AP and spontaneous f_H recordings were made at the acclimation temperature of the animal (i.e. 15°C for 15N fish; 5°C for 5N and 5H fish). Then, the saline temperature was acutely changed to 10°C and the preparation allowed 30–45 min to stabilize at the new temperature before APs and spontaneous f_H were once again rerecorded. Cardiac APs were recorded with high-resistance, sharp microelectrodes (20–40 MΩ when filled with 3.0 mol l^{-1} KCl) fabricated from thin wall borosilicate glass with an internal filament (GT120TF-3; Harvard Apparatus, Holliston, MA, USA) and using a Sutter Flaming/Brown P-1000 puller (Sutter Instruments, Novato, CA, USA).

Microelectrode signals were amplified by a high-impedance amplifier (IE-251A, Warner Instruments, Hamden, CT, USA) and the force transducer output was amplified with a CP122 AC/DC strain gage amplifier (Grass Instruments). Both signals were digitized at a sampling rate of 1000 Hz and recorded to computer using a PowerLab 8/35 data acquisition system (ADInstruments). Spontaneous f_H was calculated from the peak-to-peak intervals of the ventricular contractions. AP shape and APD were quantified by measuring V_{rest} and peak potential and calculating duration to 0 mV (APD_0), 50% (APD_{50}) and 90% (APD_{90}) repolarization. AP upstroke rate was calculated by dividing the difference in V_{rest} and peak potential by upstroke duration. Statistically significant differences in spontaneous f_H and AP characteristics among acclimation groups

were determined using one-way ANOVA. When appropriate, *post hoc* comparisons were determined using Student–Newman–Keuls analysis. Statistically significant differences in spontaneous f_H and AP characteristics between recordings conducted at acclimation temperature and 10°C within an acclimation group were determined using paired *t*-tests. Differences were considered statistically significant when $P<0.05$.

Assessment of autonomic cardiac control in 15N, 5N and 5H fish

Because of the small size of the Alaska blackfish, it was not feasible to assess the effect of temperature and chronic hypoxic submergence on autonomic cardiovascular control via serial intra-arterial injections of pharmaceutical agonists and antagonists of β-adrenoceptors and cholinergic muscarinic receptors in conjunction with *in vivo* recording of cardiovascular status. As an alternative, two methodological approaches were utilized to assess the effect of temperature and chronic hypoxic submergence on cardiac sympathovagal balance. Firstly, heart rate variability (HRV) was quantified for the post-surgery recording period (i.e. 0 h) and on the fifth day of recording (i.e. 120 h). The premise was that a persistent effect of the anaesthetic MS-222, which blocks parasympathetic activity and results in increased f_H (Randall, 1962), on HRV immediately post-ECG electrode implantation, and its subsequent loss by 120 h, could be utilized to deduce and compare sympathovagal balance in recovered 15N, 5N and 5H fish. HRV was quantified using HRVanalysis version 1.1 (Pichot et al., 2016). Briefly, RR intervals (RR_n) were plotted against their successive RR interval (RR_{n+1}) to produce Poincaré plots, of which the mean short-term (SD1) and long-term (SD2) variabilities were derived and the SD1/SD2 ratio calculated as an index of sympathovagal balance (Haverinen et al., 2014; Hsu et al., 2012). In resting vertebrates, the SD1 width reflects parasympathetic activity and the SD2 length reflects sympathetic activity. Therefore, Poincaré plots with an elongated shape (i.e. low SD1/SD2 ratio) reflect decreased parasympathetic activity, whereas Poincaré plots with more scatter (i.e. high SD1/SD2 ratio) indicate increased vagal activity. The second approach was to compare *in vivo* f_H with *in vitro* spontaneous f_H at the same measurement temperature.

Two-way RM ANOVA were used to determine statistically significant differences in the short-term (SD1) and long-term variability (SD2) values and the SD1/SD2 ratio within and between acclimation groups over time. When appropriate, *post hoc* comparisons were determined using Student–Newman–Keuls analysis. Differences were considered statistically significant when $P<0.05$.

Patch-clamp recording and analysis of I_{K1} and I_{Kr} from isolated ventricular myocytes

Fish were stunned by a sharp blow to the head, the spine was severed and the heart rapidly excised. The aortic bulb was cannulated and myocytes were isolated by retrograde perfusion of the heart with low-Na^+ solution containing (in mmol l^{-1}): 100 NaCl, 10 KCl, 1.2 $KH_2PO_4{\cdot}2H_2O$, 4 $MgSO_4{\cdot}7H_2O$, 50 taurine, 10 glucose and 10 Hepes at pH 6.9, supplemented with proteolytic enzymes (1.5 mg ml^{-1} collagenase type IA, 1 mg ml^{-1} trypsin type IX) and fatty acid-free bovine serum albumin (1.5 mg ml^{-1}) for 30–35 min, as described previously in detail (Kubly and Stecyk, 2015; Vornanen, 1997). The ventricle was minced and triturated with a Pasteur pipette to obtain individual myocytes. Cells were stored in low-Na^+ solution at 4°C and used within 8 h from isolation.

The whole-cell voltage clamp recording of K^+ currents was performed using an Axopatch 200B amplifier, a CV-203BU headstage and ClampEx 10.2 software (Axon Instruments, Foster City, CA, USA). An aliquot of dissociated cardiomyocytes was placed into a recording chamber (RC-22C, Warner Instruments; volume by depth: 138 µl mm^{-1}) mounted on the stage of an inverted microscope (Nikon Ti-S, Tokyo, Japan) and allowed to settle. Myocytes were superfused at a rate of 1–2 ml min^{-1} with an external saline solution containing (in mmol l^{-1}): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 10 Hepes, with pH adjusted to 7.6 at 21°C with NaOH. As for the *in vitro* AP and spontaneous f_H recordings, K^+ currents were measured under normoxic conditions first at the acclimation temperature of the animal (i.e. 15 or 5°C) and subsequently at 10°C. This was done to ensure comparison among the three exposure groups under identical experimental conditions and because the level of hypoxaemia in 15H fish was unknown. The temperature of the saline solution was continuously monitored with a thermistor positioned close to the myocytes and was adjusted as required using a SC-20 dual in-line solution heater/cooler and a CL-100 bipolar temperature controller (Warner Instruments). Patch pipettes were pulled from thin-walled borosilicate glass (GC150T-7.5, Harvard Apparatus) using a Sutter Flaming/Brown P-1000 puller (Sutter Instruments) and filled with K^+-based electrode solution containing (in mmol l^{-1}): 140 KCl, 1 $MgCl_2$, 5 EGTA, 4 MgATP, 0.3 Na_2GTP and 10 Hepes with pH adjusted to 7.2 with KOH. Pipette capacitance, whole-cell capacitance and access resistance were routinely compensated.

In experiments with recording of I_{K1}, 1 µmol l^{-1} tetrodotoxin, 10 µmol l^{-1} nifedipine and 2 µmol l^{-1} E-4031 were always included in the external saline solution to prevent Na^+, Ca^{2+} currents and I_{Kr}, respectively (Haverinen and Vornanen, 2009; Kubly and Stecyk, 2015). Membrane currents were elicited from the holding potential of −80 mV every 10 s by 1 s repolarizing voltage ramps from +60 to −120 mV in the absence and presence of 1 mmol l^{-1} Ba^{2+}. I_{K1} was obtained as the Ba^{2+}-sensitive current.

I_{Kr} was elicited by a double-pulse protocol from the holding potential of −80 mV using the same external saline as for I_{K1}, but in the absence and then presence of 2 µmol l^{-1} E-4031. I_{Kr} was obtained as the E-4031-sensitive current. The double-pulse protocol consisted of an initial 2 s depolarization from −60 to +60 mV (in 20 mV steps) that was followed by a 2 s repolarization to −20 mV (Hassinen et al., 2008a; Vornanen et al., 2002). During the depolarizing pulse, activation of the I_{Kr} (erg2) channels generates an outward current, but simultaneous inactivation of some of the channels obscures the true activation of I_{Kr}. When repolarized to −20 mV, the inactivation is removed and the outward tail current (at the constant driving force) is directly related to the voltage-dependent activation of the I_{Kr} channels. Therefore, we used the peak tail current to construct current density–voltage curves of I_{Kr}.

Densities of I_{K1} and I_{Kr} (expressed as pA pF^{-1}) were calculated by dividing measured currents by the cell capacitance. Two-way ANOVA with Tukey's *post hoc* analysis were used to assess statistically significant differences in K^+ currents between acclimation groups. Statistically significant differences in maximum tail I_{Kr} density (i.e. at 60 mV) between recordings conducted at acclimation temperature and at 10°C within an acclimation group were determined using paired *t*-tests. Differences were considered statistically significant when $P<0.05$.

Quantification of ventricular gene expression of major cellular components of excitation–contraction coupling

Individual fish were killed by an overdose of MS-222 (1.0 g l^{-1} buffered with 1.0 g l^{-1} $NaHCO_3$; temperature and oxygen concentration of the MS-222 solution matched that of the

acclimation condition of the fish), and within 1 min of the cessation of ventilation, the heart was accessed by a mid-ventral lateral incision, removed and rinsed in ice-cold saline (9‰ NaCl) to eliminate any residual blood. The ventricle was then dissected from the atrium and bulbus arteriosus, blotted dry and snap-frozen in liquid N_2. Tissue was stored at −80°C until use.

Total RNA was extracted and cDNA synthesized from untreated Alaska blackfish ventricle in accordance with protocols previously outlined in detail (Couturier et al., 2019; Ellefsen et al., 2008; Stecyk et al., 2012). Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). An external RNA control, mw2060, a 2060 bp mRNA species from the cyanobacterium *Microcystis* cf. *wesenbergi* that shows no sequence homology to known vertebrate mRNA species, was added to the tissue on a per unit mass basis immediately prior to tissue homogenization to provide an external reference for qPCR quantification. The use of an external RNA control has been argued to be the most accurate method for the normalization of qPCR data (Huggett et al., 2005) and mw2060 has been shown to be a more reliable method for the normalization of qPCR data from tissues of species tolerant of low-oxygen conditions than other commonly employed normalization techniques (Ellefsen et al., 2008; Stecyk et al., 2012). Total RNA (0.5 µg) from each sample was treated with DNase I (DNA-free; Invitrogen) and subsequently reverse transcribed (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Duplicate cDNA syntheses were performed on all RNA samples to allow detection of potential technical errors. Care was taken to avoid systematic errors introduced by sample processing during RNA extraction and cDNA synthesis. All samples were handled without intermission and in a systematic, yet random order. Samples were processed in groups, wherein each group consisted of samples representing each of the three acclimation conditions. The samples within each group were processed at random.

Partial cloning of the target transcripts (Table 1) occurred as previously described in detail (Couturier et al., 2019; Melleby et al., 2020; Stecyk et al., 2012). Briefly, PCR primers were designed with Primer3 (Rozen and Skaletsky, 2000) to recognize gene regions conserved among vertebrate species, as located using GeneDoc (version 2.7, Nicholas et al., 1997) and ClustalX (version 2.1) (Thompson et al., 1997). PCR was performed on a mixture of 1:30 diluted cDNA from fish from each of the acclimation conditions (Platinum®*Taq* DNA Polymerase, Invitrogen; 94°C for 10 min, 94°C for 30 s, 48°C for 1 min, 72°C for 1 min, repeat steps 2–4 44×, 72°C for 10 min, hold 4°C). Resulting dsDNA PCR products of the correct size as determined with agarose gel electrophoresis were ligated into the pGEM-T Easy Vector System I (Promega, Madison, WI, USA) and transformed into $CaCl_2$-competent cells (TOP10 F′, Invitrogen). Positive colonies were checked for inserts of a correct size via agarose gel electrophoresis and PCR products from up to eight colonies were sequenced using T7 primers (Eurofins Genomics, DNA Sequencing Services, Louisville, KY, USA). Primers utilized for partial cloning and accession numbers for all sequences determined in the present study are listed in Table 1.

We successfully obtained partial clones of all of the target genes we aimed to measure except for *snc5a*, which encodes the type V alpha subunit of the voltage-gated Na^+ channel, and *kcne1*, which encodes the beta subunit MinK of the I_{Ks}-producing slow voltage-gated (delayed rectifier) K^+ channel. Numerous primer pairs were designed and tested to amplify a partial sequence of *scn5a*. However, the cloned consensus sequence predominantly matched the predicted mRNA sequences for the type IV alpha subunit of the voltage-gated Na^+ channel (*scn4a*) of various vertebrate species, including numerous fish species. The highest nucleotide sequence similarities (at 93.84%) were to three predicted transcript variants for the sodium channel type IV subunit alpha B of another Esociformes, the northern pike (*Esox lucius*, accession numbers XM_020041954.2, XM_020041953.2 and XM_029116319.1). By comparison, the Alaska blackfish nucleotide sequence only shared 83% identity with the *Danio rerio* cardiac voltage-gated sodium channel alpha subunit Nav1.5 sequence (*zsnc5a*; accession number DQ837300.1). We thus designated the sequence *scn4a*. For *kcne1*, no sequences were obtained despite the design and testing of numerous primer pairs. Expression of *kcne1* might have been below detection levels.

qPCR primer pairs were designed from the cloned sequences and tested as previously detailed (Couturier et al., 2019; Melleby et al., 2020; Stecyk et al., 2012). Briefly, Primer3 was used for primer design, and forward and reverse primers were targeted to either side of an exon–exon overlap when possible as a further precaution against amplifying genomic DNA (i.e. in addition to the extraction of total RNA and DNAse treatment). A minimum of three primer pairs were tested for each transcript, and amplification of the desired cDNA species by the primer pairs was verified by melting curve analyses (CFX Manager™ software, Bio-Rad) and sequencing of the primer pair products (performed as described in the preceding paragraph). The sequences, efficiencies and average quantification cycle (Cq) values obtained for the primer pairs utilized for the qPCR measurements are summarized in Table 1.

qPCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). All qPCR reactions were performed in a reaction volume of 10 µl that contained 5 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 3 µl of 1:30 diluted cDNA as the template, 1 µl of 5 mmol l^{-1} gene-specific forward primer and 1 µl of 5 mmol l^{-1} gene-specific reverse primer (i.e. final primer concentrations of 1 mmol l^{-1}). The following qPCR program was used: 95°C for 10 min, 95°C for 10 s, 60°C for 30 s, repeat steps 2–3 42×. A total of four qPCR reactions were performed on each transcript for each sample of total RNA (two qPCR reactions per transcript per cDNA synthesis).

Gene expression was quantified in relation to the mRNA external standard mw2060. Cq values were obtained for each reaction using CFX Manager™ Software (Bio-Rad) and were computed with the built-in proprietary multivariable, non-linear regression model. Priming efficiencies were calculated for each qPCR reaction using LinRegPCR software (Ruijter et al., 2009), but in the final calculations, average priming efficiencies (E_{mean}) were used, calculated separately for each tissue and primer pair from all qPCR reactions (Table 1; Čikoš et al., 2007). Then, $(E_{mean})^{Cq}$ was calculated for every reaction, as well as the ratio (R1) between $(E_{mean,mw2060})^{Cq}$ and $(E_{mean,target})^{Cq}$ in order to normalize gene expression to the expression of the external RNA control mw2060 (where target is the target gene, E is the priming efficiency and Cq is the quantification cycle).

Ventricle total RNA content (ng RNA mg^{-1} tissue) was calculated from the concentration of total RNA extracted per sample, as determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer, and tissue mass. One-way ANOVA with Student–Newman–Keuls *post hoc* tests when applicable were utilized to evaluate whether acclimation condition had a statistically significant ($P<0.05$) effect on ventricle total RNA concentration and gene expression. mw2060-normalized transcript expression was $\log_{10}$ transformed prior to statistical analysis (Hellemans and Vandesompele, 2011). In addition, a pairwise

one-way permutational multivariate analysis of variance (PERMANOVA) was conducted using PAST 3.22 (Hammer et al., 2001) to determine statistically significant ($P<0.05$) differences in the pattern of gene expression among the three acclimation conditions. Principal component analysis (PCA) was conducted to visualize gene expression patterns among the three acclimation groups. Principal components were calculated using the Factoextra package (https://CRAN.R-project.org/package=factoextra) in R (http://www.R-project.org/). Corresponding factor maps were created to demonstrate the contribution of the response variables (i.e. the 15 gene targets) to the principal components.

Data reporting

Results are presented as box plots with 10th, 25th, 75th and 90th percentiles and median value or as means±95% confidence interval (CI), unless otherwise noted. *N* represents individual animals for the *in vivo* f_H, ECG, *in vitro* spontaneous f_H and gene expression data. *N* represents the number of cells for the AP and K^+ current recordings.

RESULTS

In vivo f_H, HRV and *in vitro* f_H

f_H of 15N, 5N and 5H fish slowed progressively following ECG electrode implantation to rates that subsequently remained stable for the remainder of the 5 day recording period by 24 h in 5H fish and by 48 h in 15N and 5N fish (Fig. 1A, Table 2). At each recording time, f_H of 5N fish was approximately half that of 15N fish (Fig. 1A, Table 3). 5H fish did not display hypoxic bradycardia. Resting f_H of 5H fish did not statistically differ from that of 5N fish from 72 h to 120 h of the 5 day recording period (Fig. 1A, Table 2).

No interaction was detected between acclimation group and measurement time for any of the HRV variables quantified, but measurement time was found to have a significant effect on HRV (Table 2). Concomitant with the slowing of *in vivo* f_H post-ECG electrode implantation in all acclimation groups, short-term (SD1) and long-term (SD2) variability were greater at 120 h than at 0 h. However, the increases in SD1 and SD2 were not proportional. Consequently, the SD1/SD2 ratio increased in all acclimation groups from 0 h to 120 h, indicating a change in sympathovagal balance towards increased vagal activity.

Spontaneous f_H of 5N and 5H preparations did not differ when recorded at 5°C and the rates were approximately one-third that of 15N preparations recorded at 15°C (Fig. 1B, Table 3). Acute warming increased spontaneous f_H of 5N and 5H preparations, whereas acute cooling slowed spontaneous f_H of 15N preparations such that no statistical differences existed among the three acclimation groups at 10°C (Fig. 1C, Table 3). For all acclimation groups, spontaneous f_H recorded at acclimation temperature was faster than *in vivo* f_H, indicating the presence of a tonic parasympathetic vagal inhibitory tone in the live fish.

ECG

Cold acclimation in normoxia induced prolongation of P-wave duration, PR interval, QRS duration and QT duration, with the effects being more pronounced for PR interval and QT duration (Q_{10} values near 2) than for P-wave duration and QRS duration (Q_{10} values near 1) (Fig. 2, Table 3). Chronic hypoxic submergence did not affect P-wave duration, PR interval and QRS duration compared with 5N fish (Fig. 2A–C). However, strikingly, the QT duration of 5H fish was 33% less than the QT duration of 5N fish, indicating a shortening of the average duration of the ventricular AP with prolonged hypoxic submergence (Fig. 2D).

AP shape and duration, I_{K1} and I_{Kr}

Ventricular V_{rest} and, correspondingly, the physiologically relevant outward I_{K1} were insensitive to acute and chronic temperature change, as well as chronic hypoxic submergence at 5°C. V_{rest} and outward I_{K1} did not differ between 15N, 5N and 5H fish when measured at acclimation temperature (Figs 3A and 4A). The two parameters were also unaltered following acute exposure to 10°C within all three exposure groups and outward I_{K1} density did not differ between 15N, 5N and 5H fish at 10°C (Fig. 4B). However, the culmination of the minor (~4 mV), statistically insignificant variations in V_{rest} that occurred in each exposure group with acute temperature change resulted in V_{rest} of 5H fish being more negative than that of 15N and 5N fish at 10°C (Fig. 3B).

Quantitatively consistent with the prolongation of QT duration observed *in vivo* with cold acclimation in normoxia (Fig. 2D), ventricular APD_{90} was 2.6 times longer *in vitro* in 5N ventricular preparations than in 15N preparations when measured at acclimation temperature (Fig. 3A). The prolongation of APD in 5N fish reflects that maximum tail I_{Kr} density was not upregulated with cold acclimation in normoxia (Fig. 4C,D). Rather, temperature coefficients for the effects of chronic and acute temperature change on I_{Kr} indicate that the current was largely modulated by acute and perhaps direct temperature effects (Table 3). Indeed, when measured at 10°C, I_{Kr} density at positive potentials did not differ between myocytes from 15N and 5N fish (Fig. 4D).

In line with the shortening of QT duration observed *in vivo* in 5H fish (Fig. 2D), chronic hypoxic submergence induced a pronounced shortening of ventricular APD. APD_{50} and APD_{90} were 20–29% shorter in 5H than in 5N ventricle when measured at 5°C and 10°C (Fig. 4). Correspondingly, I_{Kr} density at positive potentials was 1.4- to 1.7-fold greater in 5H than in 5N ventricular myocytes when measured at 5°C and 10°C (Fig. 4C,D).

Acute warming shortened APD_{50} and APD_{90} of 5N and 5H ventricles, with Q_{10} values above 2 (Fig. 3, Table 3). By contrast, ventricular APD was not prolonged in 15N ventricles with acute cooling, despite maximum outward I_{Kr} density decreasing with a Q_{10} of 2.1 across the same temperature range (Fig. 3, Table 3). The discrepancy may indicate that an unknown outward K^+ current that was not investigated in this study is activated and limits prolongation of APD in ventricle of 15N fish upon acute exposure to cold temperature. The current would have to be suppressed with cold acclimation in normoxia to account for the prolongation of APD recorded for 5N fish.

Ventricle total RNA content

Acclimation condition did not affect ventricular total RNA content per mg tissue. Ventricular total RNA content was 628.9±68.2, 474.7±59.1 and 614.3±70.5 ng mg^{-1} for 15N, 5N and 5H fish, respectively.

Gene expression

Only two of the 15 genes investigated exhibited a change in ventricular expression with acclimation to 5°C from 15°C in normoxia. Gene expression of *kcnj3* (encodes the G protein-activated inward rectifier K^+ channel 1, $K_{ir}3.1$) and *slc8a1* (encodes the Na^+/Ca^{2+} exchanger, NCX), was 2.6-fold and 3.2-fold greater, respectively, in 5N than in 15N fish (Fig. 5G,L). By contrast, chronic hypoxic submergence induced numerous and substantial changes in ventricular gene expression (Fig. 5). Consequently, 5H fish exhibited a statistically distinct gene expression pattern compared with that of 15N and 5N fish (Fig. 6). Specifically, gene expression of the background inward rectifier K^+ channel pore-forming alpha subunits $K_{ir}2.2$ (*kcnj12*) and $K_{ir}2.4$ (*kcnj14*) was 7.4-

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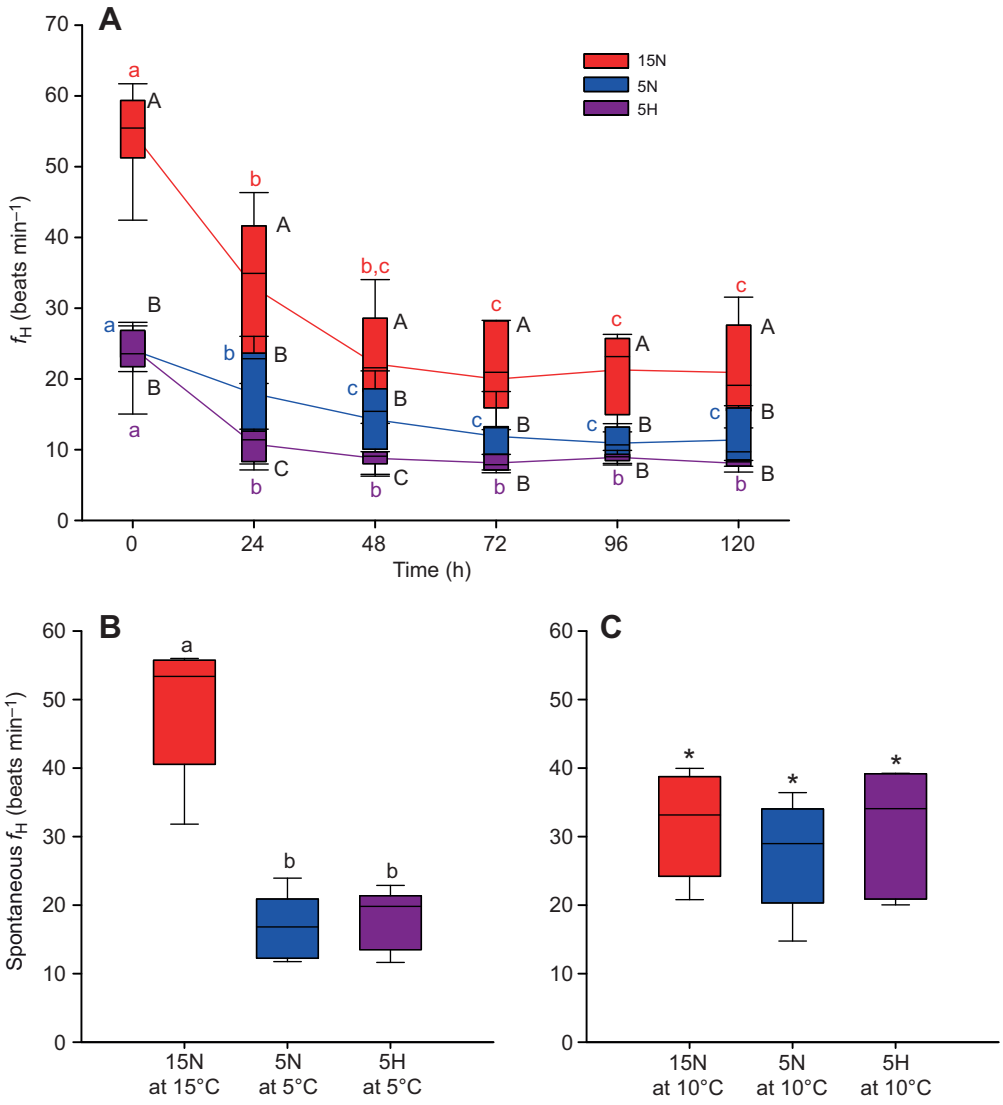

Fig. 1. *In vivo* and *in vitro* spontaneous heart rate (f_H) of 15N, 5N and 5H Alaska blackfish. (A) Box plots showing the 10th, 25th, 75th and 90th percentiles and median value of *in vivo* f_H of 15N, 5N and 5H fish (see Materials and Methods for treatment details) during the 5 day period following electrocardiogram (ECG) electrode implantation. Dissimilar lowercase letters indicate statistically significant differences (P<0.05) between time points within an acclimation group. Dissimilar uppercase letters indicate statistically significant differences (P<0.05) between acclimation groups at a given time point. Two-way RM ANOVA with Student–Newman–Keuls *post hoc* test. N=4–8 (15N), 7 (5N) and 6 (5H). (B,C) Box plots showing the 10th, 25th, 75th, and 90th percentiles and median value of *in vitro* spontaneous f_H of cardiac tissue preparations from 15N, 5N and 5H fish recorded at the acclimation temperature of the fish (B) and at the common temperature of 10°C (C). Dissimilar lowercase letters indicate statistically significant differences (P<0.05; one-way ANOVA with Student–Newman–Keuls *post hoc* test) between acclimation groups. Asterisks demarcate statistically significant differences (P<0.05; paired *t*-test) between recordings conducted at acclimation temperature and 10°C within an acclimation group. N=5 (15N), 7 (5N) and 6 (5H).

fold and 2.9-fold greater, respectively, in 5H than in 5N fish (Fig. 5B,C). Consistent with the upregulated I_{Kr} in ventricular myocytes of 5H fish, gene expression of the most prominently expressed rapid delayed rectifier K^+ channel subunit, K_v11.2 (*kcnh6*), was 3.5-fold greater in ventricle of 5H fish than in 5N fish (Fig. 5D). Similarly, gene expression of the G protein-activated inward rectifier K^+ channel subunits K_{ir}3.1 (*kcnj3*) and K_{ir}3.4 (*kcnj5*) was 2.4-fold and 3.1-fold higher, respectively, in ventricle of 5H fish than in 5N fish (Fig. 5G,H). Additionally, 5H fish exhibited increased ventricular gene expression of the L-type Ca^{2+} channel Ca_v1.2 (*cacna1c*; 2.9-fold), as well as for the proteins involved in sarcoplasmic reticulum (SR) Ca^{2+} cycling, namely ryanodine receptor-Ca^{2+} release channel (*ryr2*; 3.3-fold), the SR Ca^{2+}-ATPase SERCA2 (*atp2a2*; 10.3-fold) and phospholamban, a regulator of SERCA2 (*pln*; 3.0-fold) (Fig. 5K,M,N,O). However, the *pln*/*atp2a2* gene expression ratio was unaffected by acclimation condition (Fig. 5P). Gene expression of the Na^+/K^+-ATPase was greater in ventricle of 5H fish than in 15N but not 5N fish (Fig. 5I).

Table 2. *In vivo* heart rate (f_H) and heart rate variability (HRV) of 15N, 5N and 5H Alaska blackfish, 0 and 120 h after instrumentation with ECG electrodes

Acclimation group	Time (h)	f_H (min^{-1})	SD1 (ms)	SD2 (ms)	SD1/SD2 ratio
15N	0	54.4±5.1^a	26±7	79±16	0.33±0.07
	120	20.9±7.2^{A,*}	239±89*	530±223*	0.46±0.09*
5N	0	24.0±4.0^b	75±69	231±140	0.30±0.16
	120	11.4±3.3^{B,*}	457±290*	878±471*	0.51±0.11*
5H	0	24.1±3.0^b	60±56	212±177	0.26±0.11
	120	8.0±0.7^{B,*}	482±62*	1278±558*	0.43±0.17*

For each variable, dissimilar lowercase letters indicate statistically significant differences (P<0.05) between acclimation groups at 0 h, dissimilar uppercase letters indicate statistically significant differences (P<0.05) between acclimation groups at 120 h and asterisks indicate a statistically significant difference (P<0.05) between 0 h and 120 h within an acclimation group (two-way RM ANOVA with Student–Newman–Keuls *post hoc* analysis when appropriate). SD1, short-term variability; SD2, long-term variability. Values are means±95% confidence interval (CI). N=6 for all acclimation groups.

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Table 3. Q_{10} temperature coefficients for measured parameters

Acclimation group	Temperature range (°C)	*In vivo* f_H	Spontaneous *in vitro* f_H	P-wave duration*	PR interval*	QRS duration*	QT duration*	AP upstroke rate	APD_{90}*	Maximum tail I_{Kr}
15N–5N	15–5	1.8	2.9	1.2	1.7	1.2	2.7	2.5	2.5	1.9
15N	15–10	n/a	2.4	n/a	n/a	n/a	n/a	0.7	1.5	2.1
5N	5–10	n/a	2.5	n/a	n/a	n/a	n/a	0.5	2.7	2.3
5H	5–10	n/a	3.0	n/a	n/a	n/a	n/a	2.2	2.4	1.9

AP, action potential; APD_{90}, action potential duration to 90% repolarization. *Q_{10} calculated from reciprocal values.

DISCUSSION

The objective of the present study was to conduct a comprehensive, integrative examination of the cardiophysiological responses of the Alaska blackfish to the ambient temperature and oxygen conditions it experiences in winter. In this regard, the present study is the first to examine the cardiophysiological responses of an air-breathing fish to prolonged cold exposure, as well as to a chronic exposure to aquatic hypoxia without access to atmospheric oxygen. By comparison, past investigations, albeit with tropical species that are not restricted from air access for weeks to months in nature, have utilized hypoxia exposure times (without air access or with hypoxic or anoxic atmosphere) ranging from 15 min to 1 h at much higher temperatures (reviewed by Stecyk, 2017). Consistent with prior investigations of the effect of acclimation to cold temperature in normoxia on the cardiac, metabolic and respiratory physiology of normoxic Alaska blackfish (Kubly and Stecyk, 2015, 2019; Lefevre et al., 2014), the present findings provide additional support to the notion that the Alaska blackfish displays a unique mix of cold-compensatory, down-regulatory and acute and perhaps direct cardiophysiological responses to cold acclimation. Compared with 15N fish, 5N fish exhibited compensation of P-wave duration, QRS duration and an unaltered outward I_{K1} density, down-regulation of

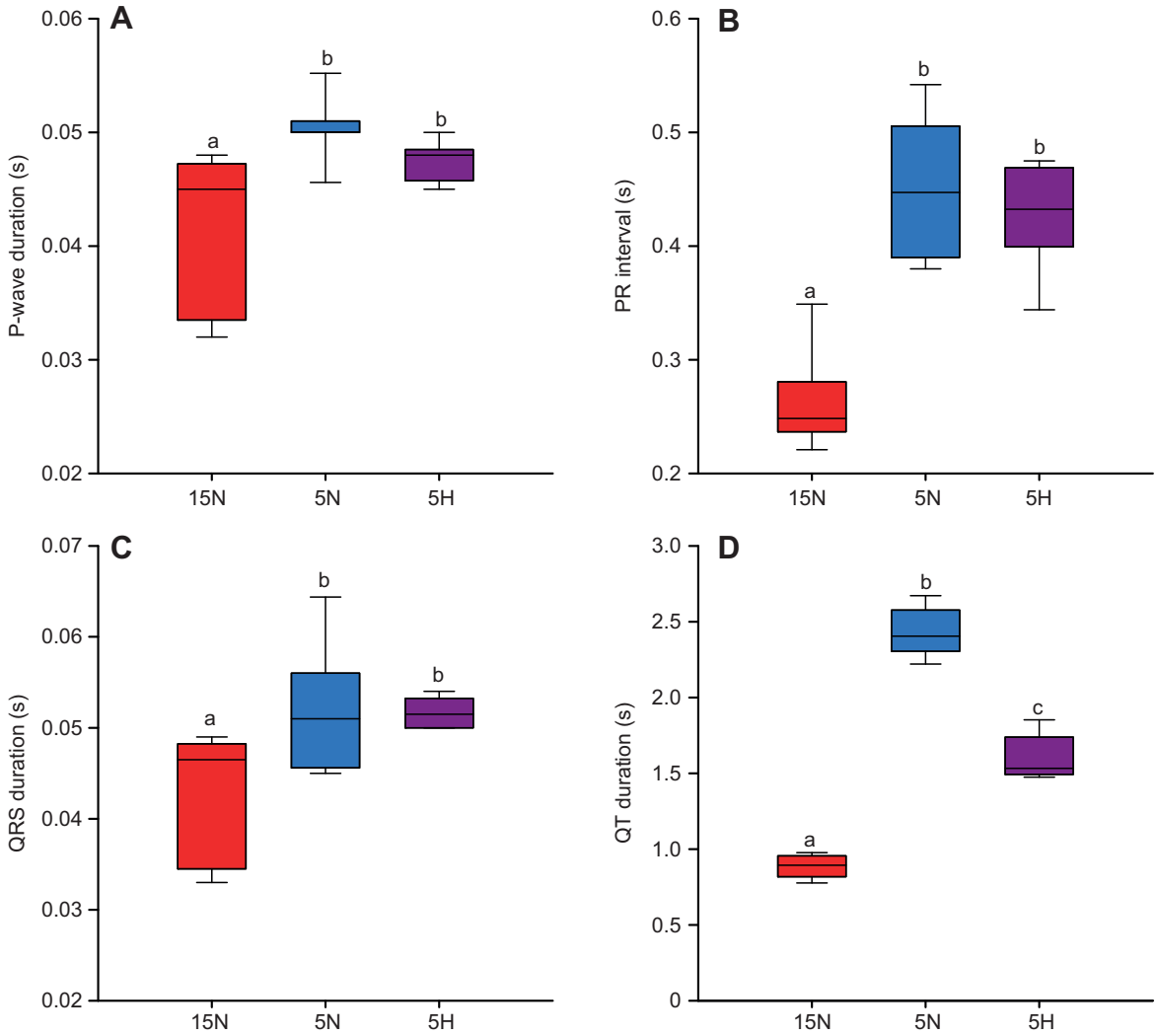

Fig. 2. ECG parameters. Box plots showing the 10th, 25th, 75th and 90th percentiles and median value of (A) P-wave duration, (B) PR interval, (C) QRS duration and (D) QT duration of 15N, 5N and 5H fish. Dissimilar lowercase letters indicate statistically significant differences (P<0.05; one-way ANOVA with Student–Newman–Keuls *post hoc* test) between acclimation groups. N=6 for all acclimation groups.

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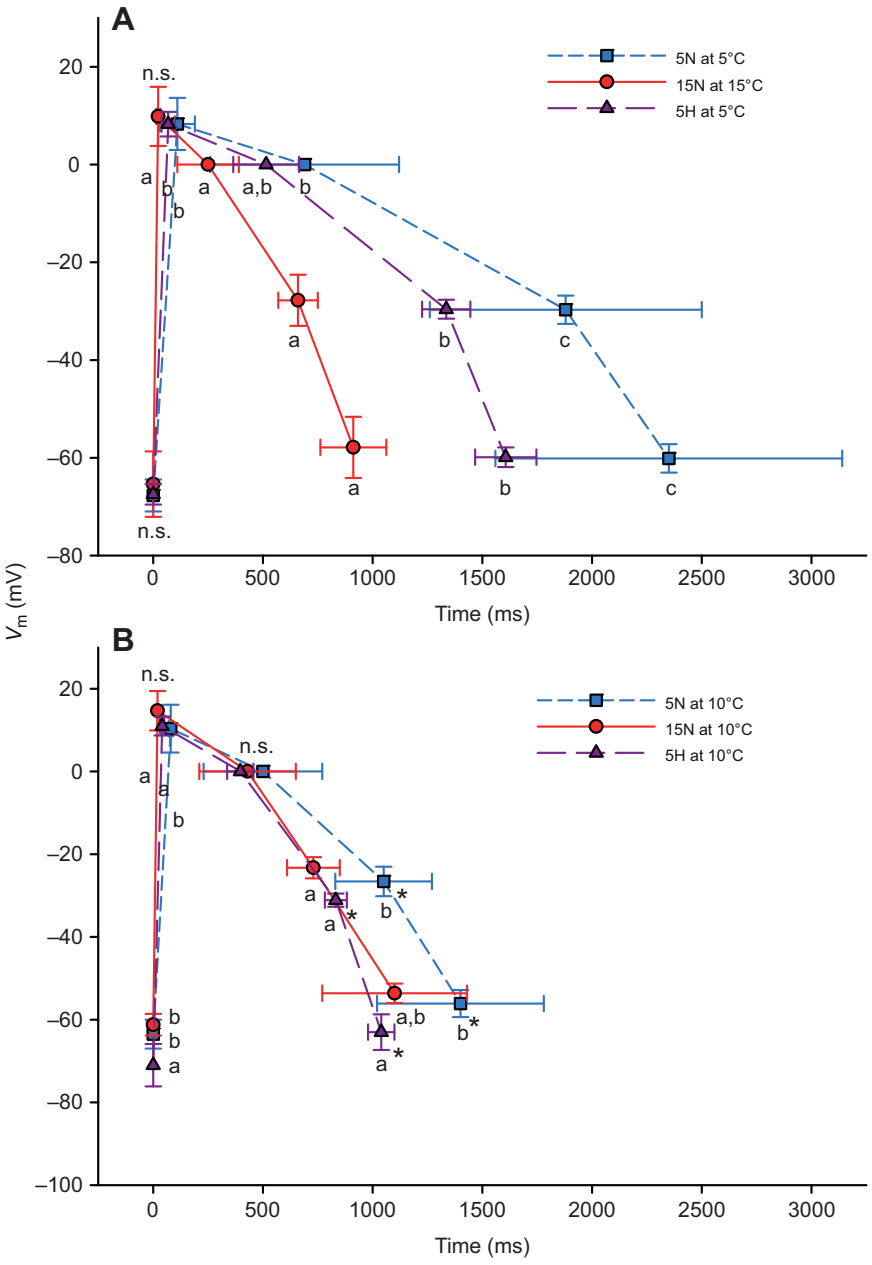

Fig. 3. Ventricular action potential (AP) shape and duration (APD). Graphical representations of ventricular APs recorded *in vitro* from spontaneously beating cardiac tissue preparations of 15N, 5N and 5H fish at (A) the acclimation temperature of the fish and (B) the common temperature of 10°C. V_m, membrane potential. Dissimilar lowercase letters indicate statistically significant differences (P<0.05; one-way ANOVA with Student–Newman–Keuls *post hoc* test) between acclimation groups for resting membrane potential, upstroke rate, peak potential and APD_0, APD_{50} and APD_{90} (duration to 0 mV, 50% and 90% repolarization, respectively); n.s., not statistically different. Asterisks demarcate statistically significant differences (P<0.05; paired t-test) between recordings conducted at acclimation temperature (A) and 10°C (B) within an acclimation group. Values are means±95% confidence interval (CI). N (cells/preparations)=5–6/5 (15N), 6–8/5 (5N) and 10–13/6 (5H).

QT duration, AP upstroke rate and APD and reductions of *in vivo* and *in vitro* f_H and maximum tail I_{Kr} density that charted acute and perhaps direct effects of temperature. Surprisingly, many of the cardiophysiological responses displayed by 5H fish were similar to the modifications of cardiac physiology exhibited by cold-active species with cold acclimation (reviewed by Vornanen, 2016, 2017). While some responses (i.e. shortened APD via up-regulation of I_{Kr}) could theoretically contribute to ATP savings by limiting Ca^{2+} influx into the cytosol, the maintained *in vivo* f_H, unaffected outward I_{K1} density and enhanced gene expression of the Na^+/K^+-ATPase (*atp1a1*), as well as channels, exchangers and proteins involved in trans-sarcolemmal and SR Ca^{2+} cycling, compared with 5N fish suggest that, contrary to our hypothesis, Alaska blackfish exposed to chronic hypoxic submergence prioritize the continuation of cardiac performance to support an active lifestyle over reducing cardiac ATP demand, at least at the level of aquatic hypoxia utilized in the present study.

In vivo f_H and insights into autonomic cardiac control in 15N, 5N and 5H Alaska blackfish

The depression of *in vivo* f_H with acclimation to 5°C from 15°C in normoxia is qualitatively and quantitatively similar to the reductions of *in vivo* f_H displayed by the anoxia-tolerant crucian carp (*C. carassius*) and the severely hypoxia-tolerant common carp (*Cyprinus carpio*) with cold acclimation (Matikainen and Vornanen, 1992; Stecyk and Farrell, 2002, 2006; Stecyk et al., 2004b; Tikkanen et al., 2017; Vornanen, 1994; Vornanen and Tuomennoro, 1999). However, the bradycardia contrasts with the positive thermal compensation and relatively high *in vivo* f_H displayed by cold-active fishes acclimated to cold temperature, including the burbot (*Lota lota*) (Tiitu and Vornanen, 2002), navaga cod (*Eleginus navaga*) (Hassinen et al., 2014) and rainbow trout (*Oncorhynchus mykiss*) (Aho and Vornanen, 2001). While the slower *in vivo* f_H of Alaska blackfish at 5°C would contribute to ATP conservation in anticipation of winter hypoxia and restricted air access, the Q_{10} near 2

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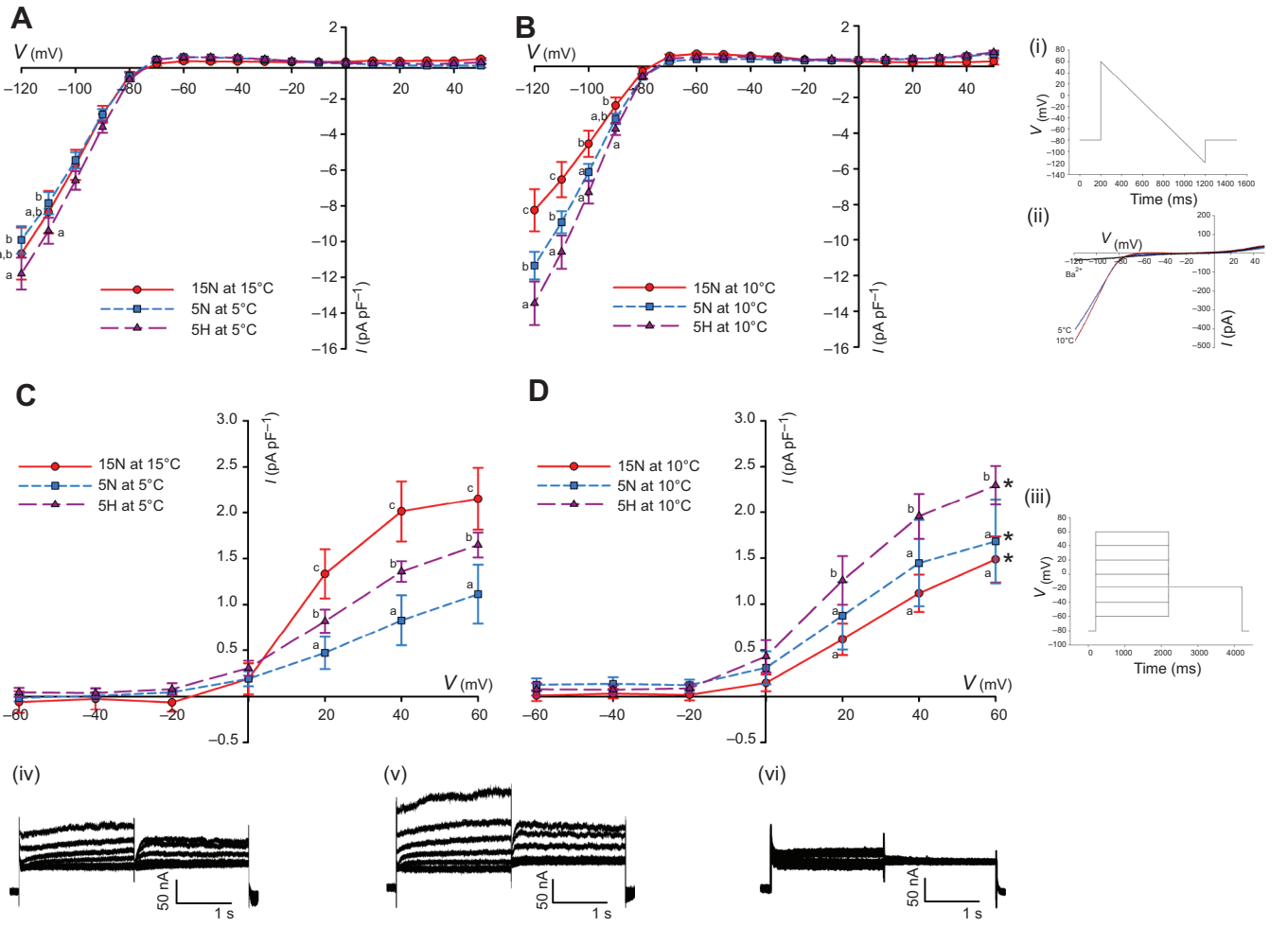

Fig. 4. I_{K1} and I_{Kr}. Current density–voltage curves of I_{K1} (A,B) and maximal tail I_{Kr} (C,D) in ventricular myocytes of 15N, 5N and 5H fish recorded at the acclimation temperature of the fish (A,C) and at the common temperature of 10°C (B,D). (i) Ramp protocol utilized to elicit I_{K1}. (ii) Original unfiltered traces of I_{K1} in one representative ventricular myocyte from a 5H fish recorded at 5 and 10°C. The trace recorded in the presence of 1 mmol l^{-1} Ba^{2+} (at 10°C) shows the leakage current not related to I_{K1}. (iii) Square-pulse protocol utilized to elicit I_{Kr}. (iv–vi) Original unfiltered traces of I_{Kr} in one representative ventricular myocyte from a 5H fish recorded at (iv) 5°C and 10°C in the absence (v) and presence (vi) of 2 µmol l^{-1} E-4031. Dissimilar lowercase letters indicate statistically significant differences (P<0.05; two-way RM ANOVA with Tukey's *post hoc* test) between acclimation groups at a given voltage. For maximum tail I_{Kr} density, asterisks demarcate statistically significant differences (P<0.05; paired t-test) between recordings conducted at acclimation temperature and 10°C within an acclimation group. Values are means±95% CI. N (cells/animals)=10/6 (15N), 12/6 (5N) and 11/6 (5H) for I_{K1}, and N (cells/animals)=13/6 (15N), 15/6 (5N) and 11/6 (5H) for I_{Kr}.

for the effect of acclimation temperature on *in vivo* f_H in normoxia is not indicative of the Alaska blackfish entering a hypometabolic state beyond that expected from direct temperature effects on physiological processes with cold acclimation. Indeed, the magnitude of the bradycardia with cold acclimation parallels the decreased total oxygen consumption (i.e. from air and water; Q_{10} of 2.2) of 5N compared with 15N Alaska blackfish (Lefevre et al., 2014). Based on the Fick equation, the similar temperature coefficients for the decreases in *in vivo* f_H and total oxygen consumption with acclimation to 5°C suggests that cardiac output also decreases with cold acclimation in Alaska blackfish and is not compensated for by an increase in stroke volume. However, future *in vivo* recordings of cardiac output are needed to confirm this prediction.

Alaska blackfish exposed to chronic hypoxic submergence did not exhibit hypoxic bradycardia. The response contrasts with the cholinergically mediated reflex slowing of f_H displayed by many water-breathing fish exposed to aquatic hypoxia (reviewed by Gamperl and Driedzic, 2009; Stecyk, 2017). With regards to the f_H response of air-breathing fish to aquatic hypoxia when air breathing is restricted, or the atmosphere is made hypoxic or anoxic, the lack of hypoxic bradycardia in 5H fish is consistent with the response of some species, but not others (reviewed by Stecyk, 2017). The lack of bradycardia in 5H fish could indicate one of three possibilities. Firstly, it could signify that the hypoxic bradycardia response does not exist in Alaska blackfish. Indeed, hypoxic bradycardia has been hypothesized to be redundant in air-breathing fishes (Farrell, 2007). Secondly, the lack of hypoxic bradycardia could indicate that the severity of aquatic hypoxia utilized in the present study was insufficient to induce hypoxaemia and/or any of the proposed direct benefits of hypoxic bradycardia to the heart (reviewed by Farrell, 2007). However, the level of hypoxia employed in the present study did induce changes in ventricular APD, I_{Kr} and gene expression compared with 5N fish. The third possibility is that the Alaska blackfish examined in the present study were acclimated to low temperature and chronic hypoxic submergence in the laboratory, rather than being exposed to these conditions naturally in the wild. Recent studies investigating the effects of temperature on fish cardiac physiology have reported that naturally occurring seasonal acclimatization induces more pronounced changes than laboratory-based thermal acclimation (Abramochkin and Vornanen, 2015;

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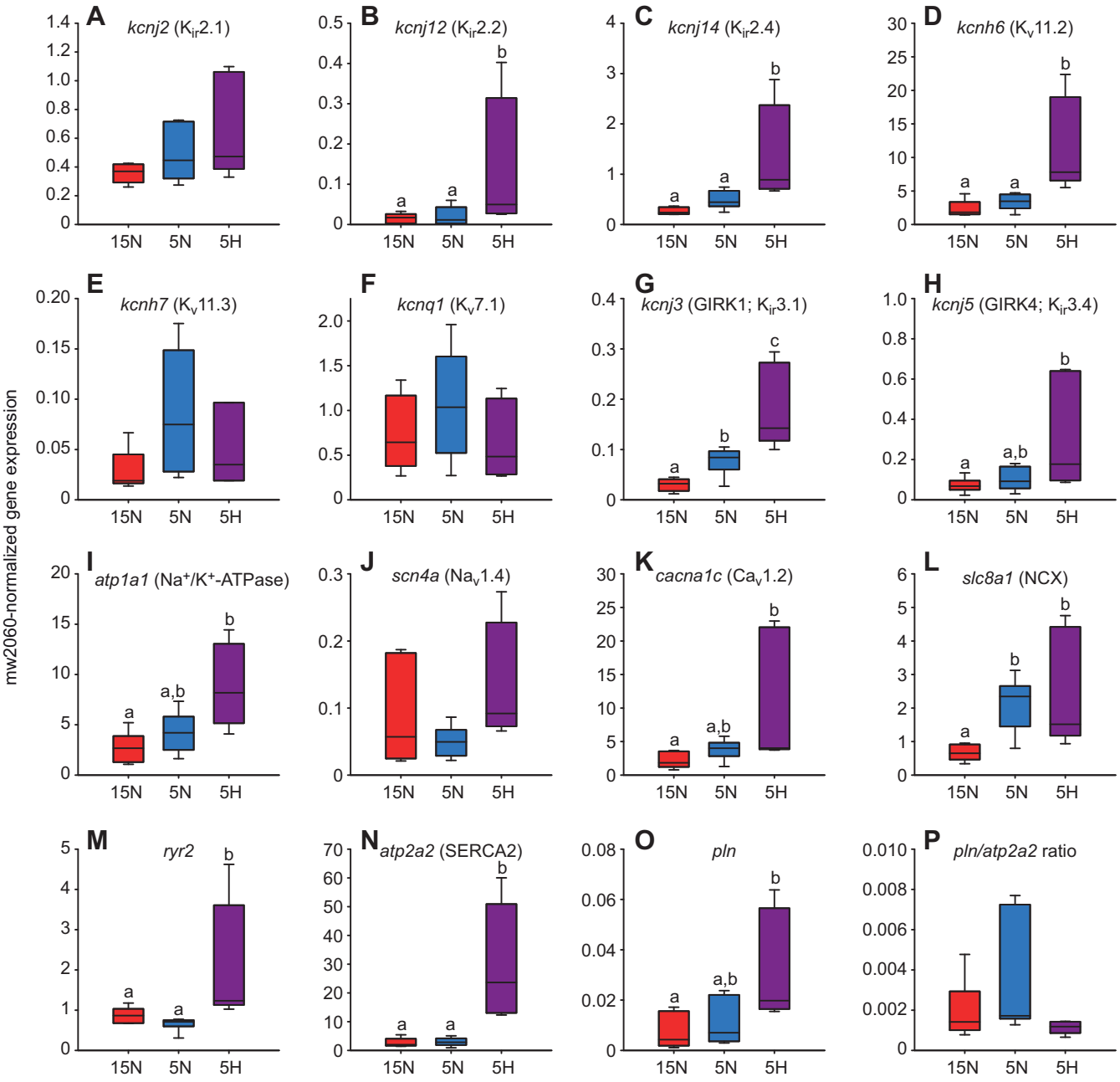

Fig. 5. Ventricular gene expression. Box plots showing the 10th, 25th, 75th and 90th percentiles and median value of gene expression (see Table 1) of proteins involved in excitation–contraction coupling in ventricle of 15N, 5N and 5H fish. (A) *kcnj2* ($K_{ir}2.1$), (B) *kcnj12* ($K_{ir}2.2$), (C) *kcnj14* ($K_{ir}2.4$), (D) *kcnh6* ($K_v11.2$), (E) *kcnh7* ($K_v11.3$), (F) *kcnq1* ($K_v7.1$), (G) *kcnj3* (GIRK1; $K_{ir}3.1$), (H) *kcnj5* (GIRK4; $K_{ir}3.4$), (I) *atp1a1* (Na^+/K^+-ATPase), (J) *scn4a* ($Na_v1.4$), (K) *cacna1c* ($Ca_v1.2$), (L) *slc8a1* (NCX), (M) *ryr2*, (N) *atp2a2* (SERCA2), (O) *pln* and (P) *pln*/*atp2a2* ratio. Dissimilar lowercase letters indicate statistically significant differences ($P<0.05$; one-way ANOVA with Student–Newman–Keuls *post hoc* test) between acclimation groups. N=5–7 (15N), 6–7 (5N) and 3–5 (5H).

Filatova et al., 2019; Hassinen et al., 2014). Indeed, paradoxically, Alaska blackfish can be caught by baited hook and line from waters covered with more than a metre of ice, with temperatures of between 2 and 4°C and with P_{O2} lower (ranging from 0.8 kPa at depth to 3.6 kPa in the water column) than their laboratory-measured critical O_2 level (Lefevre et al., 2014). However, Alaska blackfish captured in summer and autumn and then acclimated to 5°C in the laboratory did exhibit the same standard total oxygen uptake as Alaska blackfish acclimatized to low temperature for several months in nature (i.e. captured in March) (Lefevre et al., 2014). Clearly, future studies utilizing more severe levels of aquatic hypoxia and/or incorporating seasonal acclimatization are needed to differentiate the above possibilities.

In teleosts, *in vivo* f_H is largely regulated by the balance between inhibitory parasympathetic cholinergic tone and stimulatory sympathetic adrenergic tone on the pacemaker cells of the heart (Sandblom and Axelsson, 2011; Vornanen, 2017). The two methodological approaches utilized to assess autonomic cardiac control in 15N, 5N and 5H Alaska blackfish revealed a number of important implications regarding the effect of temperature and chronic hypoxic submergence on cardiac sympathovagal balance, autonomic regulation of *in vivo* f_H and the electrophysiological processes underlying intrinsic pacemaker rate in Alaska blackfish. Firstly, both approaches indicate that a tonic parasympathetic vagal inhibitory tone slows Alaska blackfish f_H under resting conditions at

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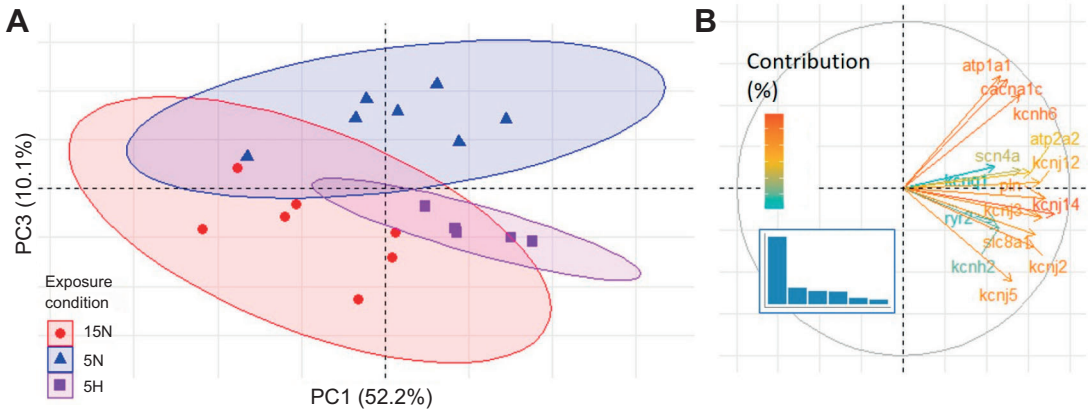

C

Acclimation group	5N	15N	5H
5N	–	0.052	**0.0017**
15N	0.052	–	**0.0014**
5H	**0.0017**	**0.0014**	–

Fig. 6. Ventricular gene expression pattern. (A) Principal component analysis (PCA) plot (PC1 versus PC3) of gene expression in ventricle of 15N, 5N and 5H fish. (B) Factor map demonstrating the contribution of the response variables (i.e. the 15 gene targets symbolized by arrows) to the principal components. The length of the arrow is directionally proportional to the contribution of variance of each gene to the total variability. The colour gradient highlights the most important genes in explaining the variation (contribution %) retained by the principal components. The scree plot of eigenvalues (inset) depicts the proportion of variance of the first six principal components. (C) *P*-values of the PERMANOVA utilized to assess differences in the pattern of gene expression among acclimation groups. Bold indicates statistically significant (P<0.05) differences.

warm and cold acclimation temperature in normoxia, as well as during prolonged hypoxic submergence. However, a caveat for the 5H *in vitro* spontaneous f_H recordings is that they were conducted under oxygenated conditions, whereas oxygen limitation can negatively affect cardiac chronotropy in vertebrates including those tolerant of low oxygen conditions (reviewed by Overgaard et al., 2007; Senges et al., 1979). Thus, the *in vitro* measurements may overestimate intrinsic heart rate *in vivo* under hypoxic conditions. A preservation of cardiac inhibitory cholinergic tone during chronic hypoxic submergence in the Alaska blackfish would be consistent with the maintenance of vagal control of f_H in the anoxia-tolerant crucian carp during short-term, warm-acclimated hypoxia (Vornanen and Tuomennoro, 1999) and long-term, cold-acclimated anoxia (Stecyk et al., 2004b), as well as the common carp during prolonged severe hypoxia exposure at 5°C (Stecyk and Farrell, 2006), but would contrast with the suppression of autonomic cardiovascular controls during short-term hypoxia in the epaulette shark (Stensløkken et al., 2004) and prolonged, cold anoxia in *Trachemys scripta* (Hicks and Farrell, 2000; Stecyk et al., 2004a).

Secondly, the relatively smaller difference between *in vitro* and *in vivo* f_H at 15°C versus 5°C, as well as the greater temperature coefficient for the effect of acclimation temperature on *in vitro* spontaneous f_H than on *in vivo* f_H, indicates that vagal tone is reduced and/or that stimulatory adrenergic tone is increased with cold acclimation as a mechanism to partially alleviate the depressive effects of decreased temperature on spontaneous f_H. A decreased cholinergic tone in 5N fish would be consistent with the reduction in cholinergic tone at decreased temperature exhibited by the severely hypoxia-tolerant common carp (Labat, 1966; Stecyk and Farrell, 2006), anoxia-tolerant goldfish (*Carassius auratus*) (Cameron, 1979; Randall, 1966), cold-dormant eel (*Anguilla anguilla*) (Seibert, 1979) and sole (*Solea vulgaris*) (Sureau et al., 1989), but would contrast with the elevation of cholinergic tone with decreased temperature in cold-active rainbow trout (*O. mykiss*) (Priede, 1974; Wood et al., 1979). An increased stimulatory adrenergic tone at 5°C could be critical to supporting cardiac contractility by partially compensating for the depressive effect of temperature on peak ventricular I_{Ca} density (Kubly and Stecyk, 2015). Indeed, 5°C-acclimated Alaska blackfish ventricular myocardium exhibits a heightened inotropic responsiveness to adrenergic stimulation, which enables greater contractile force development and maximum frequency of contraction (Kubly and Stecyk, 2019).

The final implication, as revealed by the similar spontaneous f_H of 15N, 5N and 5H preparations at the common test temperature of 10°C, is that the electrophysiological processes underlying cardiac pacemaking in Alaska blackfish are not modified by cold acclimation or chronic hypoxic submergence. For the effect of acclimation temperature, the response differs from that of crucian carp, where part of the reduction in *in vivo* f_H with cold acclimation is intrinsic to the cardiac pacemaker (Matikainen and Vornanen, 1992). For the effect of oxygen limitation, the response contrasts that of *T. scripta*, in which spontaneous f_H is re-set to a lower rate with anoxia exposure (Stecyk and Farrell, 2007).

APD, K^+ currents and ventricular gene expression in 15N, 5N and 5H Alaska blackfish

In stark contrast to most other fishes, ventricular APD was prolonged in Alaska blackfish with cold acclimation in normoxia. Concurrently, I_{Kr} was not upregulated and ventricular gene expression of the rapid delayed rectifier channel subunits K_v11.2 (*kcnh6*) and K_v11.3 (*kcnh7*) was unchanged in 5N compared with 15N fish. However, the absence of upregulation of I_{Kr} with cold acclimation in Alaska blackfish is consistent with the lack of upregulation of I_{Kr} with cold acclimation in the atrium and ventricle of the closely related northern pike (Haverinen and Vornanen, 2009). Combined, the findings challenge the current paradigm that a common characteristic of the teleost heart in response to cold acclimation is a compensatory shortening of APD. Rather, for Alaska blackfish, the findings point to

a complete lack of compensation against the prolongation of APD with cold acclimation in normoxia and that acute and perhaps direct temperature effects play a predominant role in contributing to changes in I_{Kr} density.

The physiological significance of the unusual prolongation of APD in 5N Alaska blackfish may be to offset the 8-fold reduction of peak ventricular I_{Ca} density and 4-fold reduction in Ca^{2+} influx through L-type Ca^{2+} channels (LTCCs) that occurs with cold acclimation in normoxia (Kubly and Stecyk, 2015). The longer APD, particularly the prolonged plateau phase (i.e. lengthened APD_0), would keep LTCCs open for longer, thereby increasing sarcolemmal Ca^{2+} influx. Indeed, maximal developed ventricular force is temperature independent in Alaska blackfish (Kubly and Stecyk, 2019). Similarly, the upregulated Na^+/Ca^{2+}-exchanger (NCX) gene expression (*slc8a1*) with cold acclimation may represent another means by which the effect of cold temperature on ventricular Ca^{2+} cycling is counteracted. Forward-mode NCX activity removes, whereas reverse-mode NCX activity delivers Ca^{2+} to the cardiomyocyte (Eliason and Stecyk, 2021). Therefore, the increased *slc8a1* gene expression could indicate a heightened role of the NCX to supply intracellular Ca^{2+} to support ventricular contraction at cold temperature in normoxia. Indeed, the NCX contributes significant cardiac contractile Ca^{2+} in burbot, rainbow trout, crucian carp and river lamprey (*Lampetra fluviatilis*) (Birkedal and Shiels, 2007; Haverinen et al., 2014; Shiels et al., 2006; Vornanen, 1999). Alternatively, the upregulated NCX gene expression could reflect the important role the NCX plays in regulating diastolic Ca^{2+} and a need for enhanced Ca^{2+} removal at cold temperature.

Whereas Alaska blackfish ventricular APD did not shorten with cold acclimation in normoxia, chronic hypoxic submergence at 5°C induced a profound shortening of QT duration, APD_{50} and APD_{90}. These changes were associated with an upregulation of I_{Kr} and a marked increase in the gene expression of the most prominently expressed subunit (K_v11.2; *kcnh6*) of the rapid delayed rectifier potassium channel. The shortening of APD and upregulation of I_{Kr} in 5H fish could reflect an ATP-conserving mechanism in the face of oxygen limitation. In the mammalian heart, I_{Kr} is not affected by hypoxia (Hool, 2004). However, the opening of sarcolemmal ATP-sensitive K^+ (K_{ATP}) channels aids in hypoxic survival by shortening the cardiac AP, thereby diminishing the energetic ATP demand of cardiomyocytes (Zhuo et al., 2005). In *C. carassius*, while an enhanced cardioprotective sarcolemmal K_{ATP} current and shortening of APD occurs with hypoxia exposure at 21–22°C (Cameron et al., 2013; Chen et al., 2005), the response is blunted at 4°C even though the current exists (Paajanen and Vornanen, 2002, 2003). Consequently, cardiac APD (inferred from the QT interval) is lengthened during prolonged anoxia exposure at 2°C in crucian carp (Tikkanen et al., 2017). The differential response of APD to oxygen limitation between cold-acclimated *C. carassius* and mammals has been proposed to be related to the ATP savings gained from hypoxic bradycardia superseding a need for hypoxic shortening of APD (Paajanen and Vornanen, 2003; Vornanen, 2011a). In agreement with this hypothesis, APD is also prolonged in the anoxia-tolerant turtle (*T. scripta*) with anoxia exposure at 5°C, as well as the flounder (*Platichthys flesus*) with hypoxia exposure at 10°C, both of which exhibit bradycardia during oxygen deprivation (Lennard and Huddart, 1992; Stecyk et al., 2008). Thus, for 5H Alaska blackfish, which did not display hypoxic bradycardia, protection to the heart might need to be provided by the shortening of the APD via increased I_{Kr} density. Unfortunately, K_{ATP} current or channel subunit gene expression was not assessed in the present study, but its future examination would be extremely informative in testing the assumption that K_{ATP} current is not inducible at cold temperature in fishes. Congruently, the increased I_{Kr} density in 5H fish could be a mechanism to offset enhanced Ca^{2+} entry into 5H ventricular myocytes. Gene expression of proteins involved in trans-sarcolemmal and SR Ca^{2+} cycling (L-type Ca^{2+} channel, *cacna1c*; SR Ca^{2+}-ATPase, *atp2a2*; ryanodine receptor, *ryr*; phospholamban, *pln*) was substantially enhanced with chronic hypoxic submergence, whereas APD_0, a measure of the AP plateau, was unaffected by chronic hypoxic submergence.

V_{rest} and the physiologically relevant outward I_{K1} were insensitive to temperature change and chronic hypoxic submergence. In the majority of fish species examined to date, upregulation of I_{K1} with cold acclimation helps to limit APD and stabilize V_{rest} (Vornanen, 2016, 2017). However, a reduced I_{K1} in rainbow trout with cold exposure is expected to maintain cardiac excitability (Hassinen, 2007). Thus, the maintained I_{K1} across acclimation and acute temperature change in Alaska blackfish may reflect a compromise consistent with the chimeric overwintering strategy of the fish. In agreement, the gene expression profile of background inward rectifier K^+ channel pore-forming alpha subunits in Alaska blackfish ventricle partially reflects that of rainbow trout and crucian carp. Notably, *kcnj2* (Kir2.1) and *kcnj14* (Kir2.4) gene expression co-dominated in 15N and 5N Alaska blackfish ventricle, with *kcnj12* (Kir2.2) being minimal. By comparison, *kcnj2* expression predominates in the cold-active rainbow trout ventricle, whereas *kcnj14* expression prevails in the cold-dormant crucian carp ventricle (Hassinen, 2007; Tikkanen et al., 2017). The lack of upregulation of outward I_{K1} in 5N compared with 15N fish is consistent with the prolongation of ventricular APD that occurred with cold acclimation in normoxia. Nevertheless, future examination of inward voltage-gated Na^+ current in Alaska blackfish ventricle is required to fully decipher the effects of cold acclimation on cardiac excitability.

With regards to chronic hypoxic submergence, the unaltered outward I_{K1} in 5H compared with 5N fish contrasts with the marked down-regulation of I_{K1} with prolonged anoxia exposure in ventricular myocytes of 5°C-acclimated anoxia-tolerant *T. scripta* (Stecyk et al., 2007). However, the response is similar to the maintained I_{K1} in ventricular myocytes of the anoxia-tolerant crucian carp exposed to chronic hypoxia (Paajanen and Vornanen, 2003). Given that during both diastole and systole, I_{K1} creates a K^+ leakage pathway across the sarcolemma and consequently places demands on the Na^+/K^+-ATPase, the finding indicates that lowering ATP demand via 'channel arrest', a theoretical construct that predicts that long-term survival of hypothermic and/or low-oxygen conditions is facilitated by the coordinated suppression of transmembrane ion flow through ion channels and active ion transport by ion pumps (Hochachka, 1986; Lutz et al., 1985), is not a priority for cold-acclimated, hypoxic Alaska blackfish. Indeed, the upregulation of Na^+/K^+-ATPase (*atp1a1*) gene expression in 5H fish may reflect increased Na^+/K^+-ATPase activity during hypoxia exposure due to the maintained outward I_{K1}. Finally, the increased gene expression of *kcnj12* and *kcnj14* with chronic hypoxic submergence suggests that a remodelling of background inward rectifier K^+ channel pore-forming alpha subunit composition may be important for ensuring the stability of outward I_{K1} with chronic hypoxic submergence.

Concluding remarks

These findings point to the conclusion that Alaska blackfish prioritize the maintenance of cardiac activity, rather than entering severe metabolic depression to conserve ATP, when faced with restricted air access and chronic aquatic hypoxia in winter. The

results are in accord with previous measures of oxygen consumption (from water and air and in normoxia and during acute hypoxia exposure) in 15N and 5N Alaska blackfish (Lefevre et al., 2014), the behaviour of the 5H fish in captivity (i.e. continued activity, feeding and aggression towards conspecifics) and the fact that winter-acclimatized Alaska blackfish actively feed and migrate between water bodies (Haynes et al., 2014; Lefevre et al., 2014; Leppi et al., 2016). In aggregate, the findings indicate that while Alaska blackfish may be able to survive brief excursions into severely hypoxic waters, the fish likely copes with untenable levels of aquatic hypoxia by employing a hypoxia-avoidance strategy. However, future investigations utilizing more severe levels of aquatic hypoxia and/or incorporating seasonal acclimatization are required to completely exclude the possibility that the Alaska blackfish enters a hypometabolic state to survive severe levels aquatic hypoxia when air breathing is restricted.

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Competing interests
The authors declare no competing or financial interests.

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